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<b>(21) International Application Number:</b> PCT/US90/03376 <b>(22) International Filing Date:</b> 13 June 1990 (13.06.90)  <b>(30) Priority data:</b> 365,937 13 June 1989 (13.06.89) US  <b>(71) Applicant:</b> GENENCOR INTERNATIONAL, INC. [US/US]; 180 Kimball Way, South San Francisco, CA 94080 (US).  <b>(72) Inventors:</b> HEINSOHN, Henry, G. ; 413 Copeland Street, Pacifica, CA 94044 (US). LORCH, Jeffrey, D. ; 1833 Royal Avenue, San Mateo, CA 94401 (US). HAYENGA, Kirk, J. ; 1640 Bayridge Way, San Mateo, CA 94402 (US). ARNOLD, Raymond, E. ; 894 Lombard Street, San Francisco, CA 94133 (US).		<b>(74) Agent:</b> SWISS, Gerald, F.; Burns, Doane, Swecker & Mathis, Washington & Prince Streets, P.O. Box 1404, Alexandria, VA 22313-1404 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> PROCESSES FOR THE RECOVERY OF MICROBIALY PRODUCED CHYMOSIN  <b>(57) Abstract</b>  Disclosed are methods for the recovery of microbially produced chymosin. In particular, disclosed are methods for the recovery of chymosin from the fermentation beer arising from culturing microorganisms which have been engineered so as to produce chymosin. Also disclosed are methods for the selective recovery and subsequent purification of microbially produced chymosin.  <div style="text-align: center;"><b>Property of</b> <b>SemBioSys</b> <b>GENETICS INC.</b></div> <p style="text-align: center;">PEG Kait → mon ex</p>		

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**PROCESSES FOR THE RECOVERY  
OF MICROBIALY PRODUCED CHYMOSIN**

**BACKGROUND OF THE INVENTION**

1. Field of the Invention.

10        This invention relates to the recovery  
of microbially produced chymosin. In  
particular, this invention is directed to  
methods for the recovery of chymosin from the  
fermentation beer arising from culturing  
microorganisms which have been engineered so  
15        as to produce chymosin. This invention  
further relates to methods for the selective  
recovery and subsequent purification of  
microbially produced chymosin.

2. State of the Art.

20        Chymosin is a known enzyme which is  
particularly useful in the preparation of  
cheese. Until recently, almost all chymosin  
employed commercially was recovered from the  
fourth stomach of a calf, although recovery of  
25        chymosin from the stomachs of other mammals,  
such as lamb, goats, etc. has also been  
heretofore known. However, due to the recent

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decrease in calf production, such natural sources of chymosin have declined which, in turn, has provided impetus to microbial generation of chymosin. Thus, recent patents and patent applications have disclosed that chymosin can be produced by the fermentation of genetically engineered microorganisms. For example, the production of chymosin by fermentation of filamentous fungi which have been genetically modified to express and secrete chymosin, is disclosed in U.S. Patent Application of Lawlis et al., Serial No. 07/163,219, filed February 26, 1988, which is incorporated in its entirety herein by reference. Likewise, U.S. Patent No. 4,666,847, which issued on May 19, 1987 and which is incorporated herein by reference in its entirety, discloses the production of chymosin by fermentation of *E. coli* (a bacteria) which have been genetically modified to express and secrete chymosin as well as the production of chymosin by fermentation of *Saccharomyces cerevisiae* (a yeast) which has been genetically modified to express and secrete chymosin.

Because the microbial production of chymosin results in the expression of enzymes in addition to chymosin, recovery and purification of chymosin from the fermentation beer has been an ongoing problem. For example, when chymosin is produced in the manner of U.S. Serial No. 07/163,219, enzymes

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such as alpha-amylase, acid phosphatase, leu  
amino peptidase, etc., are co-produced during  
such fermentation. The presence of such  
additional enzymes in the fermentation beer  
5 imparts an additional level of difficulty in  
the recovery and purification of chymosin from  
such beers.

While numerous methods are disclosed for  
isolating enzymes from a fermentation beer,  
10 none of the references which Applicants are  
aware of specifically disclose that chymosin  
can both be recovered in extremely high  
quantities, i.e., partition coefficients  
(K) > 85, and selectively from a fermentation  
15 beer containing enzymes in addition to  
chymosin.

For example, U.S. Patent No. 4,144,130  
describes the use of (1) a mixture of a high  
molecular weight unsubstituted or substituted  
20 polyalcohol, polyether, polyvinylpyrrolidone  
or polysaccharide and an inorganic salt, (2) a  
mixture of at least two of the above high  
molecular weight polymers to recover  
intracellular enzymes from an aqueous solution  
25 into which they have been released from the  
cells. When a mixture of polyethylene glycol  
and an inorganic salt is used, the desired  
intracellular enzyme goes into the top  
polyethylene glycol layer while the cell  
30 debris and other fermentation products go into  
the lower salt-containing layer. This

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reference discloses that the partition coefficients for various enzymes recovered in the glycol layer was about 0.3 when a normal cell mass was treated, which could be  
5 increased to about 3 when frozen cells were mixed with water and disintegrated to release their enzymes. However, this reference does not teach or suggest the selective recovery of a single enzyme, let alone chymosin from a  
10 fermentation beer containing more than one enzyme.

Similarly, U.S. Patent No. 4,728,613, discloses a process for the recovery of extracellularly produced enzymes, such as  
15 protease, amylase and microbial rennet, from whole fermentation beer by using an inorganic salt in combination with a polymer selected from the group consisting of polyethylene glycol, an amine derivative of polyethylene glycol, a carboxylate derivative of  
20 polyethylene glycol, polypropylene glycol, an amine derivative of polypropylene glycol, a carboxylate derivative of polypropylene glycol, poly(ethylene glycol) ester,  
25 polyethyleneimine, trimethylamino-polyethylene glycol, polyvinyl alcohol, polyvinylpyrrolidone and mixtures thereof. While the examples of this reference disclose achieving partition coefficients of up to about 80 for such  
30 extracellular enzymes, this reference does not specifically disclose the recovery of chymosin from a polyethylene glycol/salt mixture nor

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does it disclose the selective recovery of chymosin from a fermentation beer containing more than one enzyme.

5        Likewise, Kula et al., "Purification of  
Enzymes by Liquid-Liquid Extraction",  
describes numerous methods for the  
purification of enzymes by liquid-liquid  
extraction. Among numerous methods disclosed,  
10       Kula et al. disclose that the addition of a  
polyethylene glycol/inorganic salt mixture to  
an aqueous solution containing the enzyme will  
form a two phase system wherein the  
polyethylene glycol phase will contain the  
enzyme. Kula et al. further disclose at page  
15       111 that the phase forming polymer  
(polyethylene glycol) can be removed from the  
enzyme by adsorption of the enzyme onto ion  
exchangers; washing away of the phase forming  
polymer; and the subsequent recovery of the  
20       enzyme. However, this reference does not  
specifically disclose the recovery of chymosin  
using a polyethylene glycol/salt mixture nor  
does it disclose the selective recovery of  
chymosin from a fermentation beer containing  
25       more than one enzyme.

On the other hand, U.S. Patent No.  
4,508,825 discloses that extracellular  
protease and amylase co-produced during the  
fermentation of a microorganism capable of  
30       producing them are separated by the addition  
of polyethylene glycol and a cationic

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5       epihalohydrin/polyamine copolymer or dextran  
polymer to the fermentation medium and  
allowing the polymers to phase separate to  
form a protease rich phase and an amylase rich  
phase.

10       Also, U.S. Patent No. 4,591,563,  
discloses a process for the simultaneous  
purification and concentration of the dextran-  
sucrase enzyme from the culture medium on  
sucrose. In particular, the disclosed method  
involves the addition of a polyether such as  
polyethylene glycol so as to form two phases;  
the first a heavy dextran-rich phase that  
contains the concentrated and purified  
15       dextran-sucrase enzyme, and the second a  
lighter polyether-rich phase that contains  
contaminating enzymatic activities, which is  
eliminate.

20       In view of the above, it is apparent that  
the cited art does not disclose recovery of  
microbially produced chymosin from a  
fermentation beer using a two phase liquid-  
liquid extraction method having partition  
coefficients for chymosin of greater than  
25       about 85.

30       It is further apparent that the cited art  
does not disclose the selective recovery of  
chymosin from a fermentation beer containing  
fermentation enzymes in addition to chymosin,  
i.e., most of the chymosin is recovered in one



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layer whereas most of the other fermentation enzymes are recovered in the other layer.

On the other hand, industrial or commercial scale production of chymosin by  
5 microbial activity and its subsequent purification is greatly facilitated by large partition coefficients for chymosin in a liquid-liquid two phase system and also by the selective recovery of chymosin from other  
10 fermentation enzymes contained in the beer when such two phase systems are employed.

Accordingly, it is an object of this invention to provide efficient processes for the recovery of microbially produced chymosin  
15 from aqueous mixtures of enzymes produced by fermentation or other microbial activity and particularly for commercial scale production of chymosin.

It is a further object of this invention to provide a recovery process for microbially  
20 produced chymosin using a liquid-liquid two phase system having partition coefficients for chymosin of greater than about 85.

It is still a further object of this invention to provide a recovery process for  
25 microbially produced chymosin using a liquid-liquid two phase system which provides for the selective recovery of chymosin from

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other polypeptides contained in the  
fermentation beer including other enzymes.

5 It is a further object of this invention  
to provide a process for the recovery and  
purification of microbially produced chymosin.

10 These and other objects are achieved by  
the present invention as evidenced by the  
attached summary of the invention, detailed  
description of the invention, examples, and  
claims.

#### SUMMARY OF THE INVENTION

15 In one aspect, this invention is a method  
for recovering microbially produced chymosin  
from an aqueous fermentation beer additionally  
containing fermentation polypeptides which  
comprises adding to the fermentation beer an  
effective amount of polyethylene glycol (PEG)  
and an inorganic salt so as to form a two  
20 phase system, allowing the fermentation beer-  
polyethylene glycol-inorganic salt mixture to  
separate into a chymosin-rich, fermentation  
polypeptide-poor polymer phase and a chymosin-  
poor, fermentation polypeptide-rich salt  
phase, and recovering the chymosin-rich,  
25 fermentation polypeptide-poor polymer phase.

Surprisingly, this recovery method  
provides for selective recovery of chymosin  
from the fermentation polypeptides found in

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the fermentation beer and also provides for partition coefficients for chymosin recovery which are greater than about 85 and preferably greater than about 100.

5           In general, the pH of the fermentation beer can be any pH at which the chymosin is stable, i.e., about 6.5 or less. However, in a preferred embodiment, it has been found that the use of lower pHs, i.e., pH 3 or less and  
10           preferably from about pH 2 to about 2.5, in the fermentation beer result in higher partition coefficients (higher selectivity) for the separation of chymosin into the polyethylene glycol phase, as high as 1000 or  
15           more, as compared to the use of pHs of from above 3 to about 6.5.

          Accordingly, a preferred method aspect of the present invention relates to a method for recovering microbially produced chymosin from  
20           an aqueous fermentation beer additionally containing fermentation polypeptides which comprises adjusting the pH of the fermentation beer to less than about 3, then adding to the fermentation beer an effective amount of  
25           polyethylene glycol (PEG) and an inorganic salt so as to form a two phase system, allowing the fermentation beer-polyethylene glycol-inorganic salt mixture to separate into a chymosin-rich, fermentation polypeptide-poor  
30           polymer phase and a chymosin-poor, fermentation polypeptide-rich salt phase, and

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recovering the chymosin-rich, fermentation polypeptide-poor polymer phase.

- 5 In a further aspect of this invention, it has been found that chymosin can be separated from the polyethylene glycol phase by contacting the polyethylene glycol phase with an ion exchange resin under conditions wherein the chymosin binds to the resin. Under these conditions, the polyethylene glycol passes through the resin and chymosin is then recovered from the resin. Accordingly, this method aspect of the present invention relates to a method for recovering and purifying microbially produced chymosin from an aqueous fermentation beer additionally containing fermentation polypeptides which comprises
- 10 a) adding to the fermentation beer an effective amount of polyethylene glycol (PEG) and an inorganic salt so as to form a two phase system,
- 20 b) allowing the fermentation beer-polyethylene glycol-inorganic salt mixture to separate into a chymosin-rich, fermentation polypeptide-poor polyethylene glycol phase and a chymosin-poor, fermentation polypeptide-rich salt phase,
- 25 c) recovering the chymosin-rich, fermentation polypeptide-poor polyethylene glycol phase,
- 30 d) contacting the chymosin-rich, fermentation polypeptide-poor polyethylene glycol phase with an ion exchange resin under

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conditions wherein the chymosin binds to the resin and the polyethylene glycol passes through the resin; and

5 e) recovering the chymosin from the resin.

In this aspect of the invention and for the reasons noted above, it is preferred that the pH of the initial fermentation beer be adjusted to about 3 or below before adding the polyethylene glycol/salt to the beer. It is also preferred that the pH remain below about 3 through the separation of the PEG phase and the contacting of the PEG phase with the ion exchange resin.

15 **DETAILED DESCRIPTION OF THE INVENTION**

The separation of various enzymes from aqueous solutions using polymers such as polyethylene glycol of various molecular weights in combination with other polymers, e.g., dextran, or inorganic salts is known in the art. However, this invention is directed in part to the unexpected discovery that extremely high partition coefficients for the extraction of microbially produced chymosin, particularly extracellularly produced chymosin, can be achieved by adding a sufficient amount of both polyethylene glycol and an inorganic salt to the fermentation beer so as to form a two phase system. Under these circumstances, almost all of the chymosin is

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partition into the polyethylene glycol phase. This is evidenced by partition coefficients for chymosin in the polyethylene glycol phase of greater than about 85 and preferably  
5 greater than about 100.

Further, this invention is also directed to the unexpected discovery that extraction (recovery) from the fermentation beer by the addition of polyethylene glycol and an  
10 inorganic salt is highly selective for microbially produced chymosin. That is to say that this extraction process is selective for chymosin and that other polypeptides (including other enzymes) found in the  
15 fermentation beer are preferentially retained in the chymosin-poor salt phase. This latter discovery is particularly surprising in view of the fact that the art which discusses enzyme recovery from an aqueous solution via  
20 the addition of a polymer and an inorganic salt does not teach selective recovery of one enzyme from other enzymes present in the solution.

However, prior to discussing this  
25 invention in detail, the following terms will first be defined:

"Microbially produced chymosin" -- means a microbially produced polypeptide containing a sufficient homology of amino acids to  
30 naturally occurring chymosin as to possess the

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enzymatic activity of naturally occurring chymosin and having the partition characteristics of naturally occurring chymosin in the liquid-liquid two phase aqueous system described herein. Microbially produced chymosin is generally prepared by cultivating (fermenting) a host microorganism, i.e., a filamentous fungus, a bacteria, a yeast, and the like, transformed with a recombinant DNA sequence encoding all or a sufficient portion of chymosin so that the produced polypeptide possess the enzymatic activity of naturally occurring chymosin. See, for example, U.S. Serial No. 163,219 as well as U.S. Patent No. 4,666,847 for disclosure of suitable transformed host microorganisms capable of generating microbially produced chymosin.

Microbially produced chymosin can be further classified as either intracellularly or extracellularly produced. Specifically, an intracellularly produced enzyme is one which is produced and retained in the host cell during fermentation and at the completion of fermentation must be released from the cell, generally by lysing the cell. Cells can be conventionally killed and lysed by using heat. See, for instance, Wegner et al., U.S. Patent No. 4,601,986. Another method useful on certain microorganisms is to change the osmotic pressure which causes the cells to lyse. See, for instance, Aubert et al., U.S.

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Patent No. 4,299,858. Another conventional method used for lysing cells is by introduction of enzymes which break down the cell walls or membranes. Examples of this method are disclosed by Sugiyama, U.S. Patent No. 3,816,260; Kobayashi et al, U.S. Patent No. 3,890,198; and Kitamura et al., U.S. Patent No. 3,917,510. The disclosures of the above patents are incorporated herein by reference.

10           An extracellularly produced enzyme is one which the microorganism is capable of transporting across the cell wall and accordingly, is found in the fermentation beer at the completion of the fermentation without  
15 any further treatment to the cells.

"Naturally produced chymosin" -- refers to chymosin recovered from mammalian sources, including the fourth stomach of a calf.

20           "Fermentation beer" -- refers to the chymosin containing solution recovered from the fermentation of the transformed host cell (microorganism) including any subsequent treatments which are performed on the beer. Such optional treatment steps include, for  
25 example, the killing of the cells (microorganisms) in the fermentation beer prior to addition of the inorganic salt and polyethylene glycol to the fermentation beer. Killing of the microorganism is preferably  
30 accomplished in the manner described in U.S.



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Serial No. 07/365,945, which is incorporated herein by reference. Additionally, in some cases, it may be desirable to remove the cells and/or cell debris from the fermentation beer prior to addition the inorganic salt and polyethylene glycol. Such removal can be effected by a filtration step which removes most or all of the solids including the cells and/or cell debris. Likewise, in some cases, it may be desirable to adjust the pH of the beer prior to addition the inorganic salt and polyethylene glycol. Also, for intracellularly produced chymosin, it is common to remove the lysed cells and recover inclusion bodies which contain the bulk of the chymosin which after refolding, is then treated in the manner of this invention.

"Fermentation polypeptides" -- refer to the polypeptides, other than chymosin, which may be produced by the host microorganism during fermentation and which, in addition to chymosin, are contained in the fermentation beer at the time of addition of the polyethylene glycol and the inorganic salt. A major component of such fermentation polypeptides are enzymes which are co-produced during the fermentation. For example, the extracellular production of chymosin in the manner described in U.S. Serial No. 07/163,219 also results in the production of enzymes such as alpha-amylase, acid phosphatase, leu amino peptidase, etc. which accordingly are found in

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the fermentation beer at the completion of fermentation. Likewise, when chymosin is produced intracellularly, enzymes in addition to chymosin may include any extracellular  
5 enzymes produced during fermentation of the host cells as well as the intracellular enzymes released upon lysis of the host cells at the completion of fermentation so as to release chymosin.

10 "Fermentation by-products" -- refer to the non-polypeptide products contained in the fermentation beer at the time the polyethylene glycol and inorganic salt are added to the fermentation beer, including, for example,  
15 organic acids, complex carbohydrates, etc.

"Polyethylene glycol" -- refers to any molecular weight polyethylene glycol which can be used to extract chymosin in the manner of this invention. Polyethylene glycol is  
20 available in molecular weights ranging from about 400 to about 22,000. Preferred polyethylene glycol for use herein should have a molecular weight in the range from about 600 to about 12,000. A particularly preferred  
25 polyethylene glycol is PEG-8000, i.e., polyethylene glycol having a molecular weight in the range of about 8,000. The selection of the polyethylene glycol used will depend in part on the composition of the mixture from  
30 which the chymosin is to be extracted and in

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part on economics of the process, as well as other factors.

"Inorganic salt" -- refers to any inorganic salt which can be used to extract chymosin in the manner of this invention. Suitable inorganic salts include for instance, sulfate salts, phosphate salts, and the like. The sulfate salts are preferred including sodium sulfate, magnesium sulfate, ammonium sulfate, and the like. Additionally, mixtures of suitable salts can also be used as well as mixtures of such salts in combination with salt(s) such as sodium chloride, which by itself does not partition into a two phase system with polyethylene glycol but in combination with a suitable inorganic salt are known to enhance the partition coefficients of enzymes.

"Partition coefficient (K)" -- is defined by the formula

$$K = C_t / C_b$$

where  $C_t$  refers to the equilibrium concentration of the partitioned compound in the top phase and  $C_b$  refers to the equilibrium concentration of the partitioned compound in the bottom phase. Accordingly, it is apparent that the quantitative amount of partitioned compound in either phase depends on its partition coefficient as well as the volume of the phases. That is to say that if the partitioned compound has a partition

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coefficient of unity (the compound is equally partitioned in the top and bottom phases), then the phases will contain equal quantities of the partitioned compound only if the phases are of equal volume. If the top phase has 10% of the volume of the bottom phase, then when the partition coefficient is unity, the top phase will contain only 10% of the partitioned compound. In view of the above, it is further apparent that a very high partition coefficient for the partitioned compound is extremely valuable because it allows recovery of large quantities of this compound in the upper phase even when the volume of the upper phase is relatively small as compared to the bottom phase. Thus, in the present invention, very high partition coefficients allow for the use of smaller quantities of polyethylene glycol while still achieving very high recoveries of chymosin.

"Isoelectric point (IP)" -- refers to the pH at which a polypeptide will be electrostatically neutral, i.e., the polypeptide carries an equal number of positive and negative charged functionalities. The isoelectric point for chymosin is about 4.6. At a pH below its isoelectric point, chymosin will have a net positive charge; and at a pH above its isoelectric point, chymosin will have a net negative charge.

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"Ion exchange resin" -- refers to a protein compatible resinous material which is capable of electrostatically binding charged compounds. Ion exchange resins are well known in the art and include both cation and anion exchange resins.

In the practice of this invention, a solution of chymosin is contacted with an ion exchange resin under conditions wherein the chymosin will bind to the resin. Whether a cation or an anion exchange resin is employed in the present invention depends on the pH of the polyethylene glycol phase, i.e., whether the pH of the solution is above or below the isoelectric point of chymosin. Accordingly, contacting a solution containing chymosin with an ion exchange resin under conditions wherein the chymosin binds to the resin merely refers to adjusting the pH of the solution above or below its isoelectric point so that the chymosin binds to the resin employed.

The pH of the solution is generally about 6.5 or less, although pH's around the isoelectric point, i.e., pH from about 3.6 to about 5.0, are not preferred due to the low net electrostatic charge of chymosin which reduces its effectiveness in binding to the resin.

Additionally, when the polyethylene glycol phase (solution) is maintained at pH

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3.0 - 5.0, the chymosin undergoes more efficient autolysis, although autolysis is appreciable slower in the polyethylene glycol solution than in water. In any event, maintaining the aqueous polyethylene glycol phase at between 3.0 - 5.0 will result in some losses in chymosin yield due to autolysis. Accordingly, when a cation exchange resin is employed, it is preferred that the pH of the solution be maintained below about 3.0; whereas when an anion exchange resin is employed, it is preferred that the pH be maintained at above about 5.0.

Preferably, for the reasons noted above, the pH of the polyethylene glycol solution is about 3 or less, preferably less than about 3, and more preferably, from about 2 to about 2.5. When low pH's are employed, it has been further found that the fermentation by-products are readily passed through the resin whereas at higher pH's (5 and above) and when using an anion exchange resin, some of the fermentation by-products irreversibly bind to the anion exchange resin, thus shortening its useful life.

Preferred cation exchange resins for use in this invention include, for instance, IBF SP Spheredex, Pharmacia SP-Sephadex, Indion SP-2, IBF SP-Trisacryl, and the like. Preferred anion exchange resins for use in this invention include, for instance,

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IBF Q Spherox, Pharmacia Q-Sephadex, Indion Q-2, IBF Q-Trisacryl, and the like.

5       The processes of this invention are useful for recovery and/or purification of microbially produced chymosin. When recovering and purifying microbially produced chymosin, the entire fermentation beer or mixture may be used in its crude form or, if desired, the fermentation mixture can first be  
10       filtered to remove most or all of the solids then the liquid filtrate used in the processes of this invention.

15       In one aspect of this invention, microbially produced chymosin is recovered by adding to the fermentation beer an effective amount of polyethylene glycol (PEG) and an effective amount of an inorganic salt so as to form a two phase system. The resulting solution is allowed to stand so as to separate  
20       into a chymosin-rich, fermentation polypeptide-poor polyethylene glycol phase and a chymosin-poor, fermentation polypeptide-rich salt phase. The chymosin-rich, fermentation polypeptide-poor polyethylene glycol phase is  
25       then recovered by conventional techniques.

      It has been found that under these conditions, partition coefficients for chymosin in the polyethylene glycol phase of greater than 85 and preferably greater than  
30       100 are achieved. While polyethylene glycol

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5 extraction is useful for recovering chymosin  
at higher pH levels, i.e., pH 6.5 or less, it  
is more efficient as when the process is  
conducted at a pH less than about 3. At such  
10 lower pHs, partition coefficients of up to  
1000 or more can be achieved. High partition  
coefficients are particularly advantageous  
because such permit the use of smaller  
quantities of polyethylene glycol to achieve  
the desired separation of chymosin from the  
15 fermentation beer which, in turn, facilitates  
the later separation of chymosin from the  
polyethylene glycol, i.e., there is less  
polyethylene glycol to separate.

15 It has further been found that as a  
result of a single extraction, the  
polyethylene glycol phase can contain as much  
as 95% or more of the total chymosin initially  
present in the fermentation beer and contains  
20 very little, if any, fermentation polypeptides  
or fermentation by-products from the beer.  
Thus, in addition to providing a means for  
recovering substantially all of the  
microbially produced chymosin contained in the  
25 fermentation beer, this aspect of the present  
invention also provides for the selective  
recovery of chymosin from the beer. This  
selectivity for chymosin recovery allows for  
both chymosin recovery from the fermentation  
30 beer as well as separation of the chymosin  
from most of the fermentation polypeptides and  
fermentation by-products co-produced in the



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fermentation. Thus, recovery of chymosin in the manner of this invention will result in a polyethylene glycol phase having a contamination of chymosin by such fermentation polypeptides and fermentation by-products of at least about 75% less, and preferably at least about 90% less, as compared to the contamination of chymosin by such fermentation polypeptides and fermentation by-products prior to extraction, i.e., in the fermentation beer.

In another aspect of this invention, the polyethylene glycol phase containing the extracted chymosin is separated from the other phase or phases and the polyethylene glycol phase is contacted with an ion exchange resin while maintaining or adjusting the pH so that the chymosin will bind to the resin. Because the polyethylene glycol is not charged, under these conditions, it passes through the resin thus effecting purification of chymosin from the polyethylene glycol. Thus, when the isolated polyethylene glycol phase containing the extracted chymosin is contacted with the ion exchange resin under conditions where the chymosin binds to the resin, essentially all the chymosin comes out of the polyethylene glycol and is bound to the ion exchange resin and the polyethylene glycol pass through the resin column. After the initial contact, the resin is washed with either water or water and salt, preferably so not to remove

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the chymosin from the resin, to remove the remaining polyethylene glycol. Then the chymosin is eluted from the column using a salt solution and a buffer maintained at a pH which will remove the chymosin from the resin. Because of the high selectivity for chymosin recovery, it is unnecessary to use a gradient or step-wise elution of the resin because essentially the only enzyme or material which is bound by the resin from the polyethylene glycol phase and subsequently released from the resin is chymosin. Therefore, the chymosin can be eluted in one bulk step using the salt solution and raising or lowering the pH (depending of course, on whether a cation or anion exchange resin is employed) to cause the entire chymosin content bound to the resin to be eluted in one batch. Preferably, a salt is added to the eluting solution to aid in the rate or degree of elution, or in some cases, i.e., with an anion exchange resin, to effect elution of the chymosin from the resin. Salts are preferably employed with the eluting solution since chymosin is usually sold in commercial form in a salt solution and accordingly, it is convenient to incorporate the salt with the chymosin at this point.

Although higher pH levels can be used throughout the process (pHs up to about 6.5), the efficiency of the polyethylene glycol/inorganic salt mixture in extracting the chymosin from the fermentation beer and

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therefore the efficiency of the process is not as high as when low pH is maintained throughout the extraction step. Therefore, it is preferred to use low pH and accordingly, a cation exchange resin to increase the efficiency of the overall process.

When the preferred aspects of the above invention are combined by conducting the polyethylene glycol extraction at a pH of about 3 or below and contacting the separated polyethylene glycol phase with a cation exchange resin while maintaining the low pH, it has been found that a single polyethylene glycol extraction and a single-pass contact with the ion exchange resin will recover in the range of 90 to 95% of the total chymosin present in the initial fermentation beer.

On the other hand, the use of a higher pH in the polyethylene glycol extraction step as well as an anion exchange resin while maintaining the pH above chymosin's isoelectric point also provides acceptable results, although in this embodiment, the resin life is diminished by the accumulation of irreversibly bound fermentation by-products. When a higher pH is employed to extract the chymosin, this latter problem can be avoided simply by adjusting the pH of the polyethylene glycol extract to below chymosin's isoelectric point and preferably, to about pH 3.6 or below and more preferably

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about pH 3 or below, and then contacting the chymosin with a cation exchange resin.

5 In addition to the high efficiency for chymosin recovery by using the polyethylene glycol/inorganic salt mixture herein described, it has been further found that the solubility of chymosin in polyethylene glycol is apparently so high that the chymosin moves from the aqueous phase into the polyethylene glycol phase very rapidly. The time required for extraction of the chymosin into the polyethylene glycol phase is usually so short that it is not a significant process design factor. This process is therefore very efficient and economic in operation and is easily scaled up for commercial production.

20 As is apparent and regardless of the pH employed, the herein described process involves moving the chymosin from the aqueous mixture into the more hydrophobic polyethylene glycol phase. This is driven, at least in part, by the salt concentration in the non-polyethylene glycol phase or phases. If a low molecular weight polyethylene glycol is used, the polyethylene glycol phase is less hydrophobic and a higher salt concentration is necessary in the non-polyethylene glycol phase(s), which adds to the cost of operation. If a higher molecular weight more hydrophobic polyethylene glycol is used, less salt will be needed in the process, but the separation rate

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may be lower because of the high viscosity of the higher molecular weight polyethylene glycol. Thus, the process of this invention can be optimized for any particular operation by selecting the desired polyethylene glycol molecular weight, the salt concentration and other parameters which provide the desired economics. One objective is usually to minimize the time required to move the chymosin into the polyethylene glycol phase, but another objective is usually to minimize the amount of salt used to effect the transfer of essentially all of the chymosin into the polyethylene glycol phase. While the salt concentration in the non-polyethylene glycol phases can be 20% by weight or higher, usually less than about 15% is required with the appropriate polyethylene glycol. For example, with PEG-8000, about 10-13% sodium sulfate is adequate. On the other hand, the minimum inorganic salt concentration is dictated by the concentration of the salt necessary to form a 2 phase system with polyethylene glycol. However, in a preferred embodiment, the inorganic salt concentration is from about 8.5 to about 20 weight to volume percent based on the volume of the fermentation beer.

Also, preferably, the polyethylene glycol concentration employed herein is less than about 20, and more preferably less than about 15, weight to volume percent based on the volume of the fermentation beer. For

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economics and ease of later separation, as little polyethylene glycol as possible is most preferably employed.

5       The exact concentration of polyethylene glycol and inorganic salt employed herein can readily be determined by the skilled artisan.

10       After the chymosin is bound by the ion exchange resin, the polyethylene glycol phase may be recovered and reused by collecting the polyethylene glycol phase which is passed through the resin and either further treating it with chemicals, such as activated charcoal, to remove additional impurities or by directly adding it back to another batch of starting materials.

15

20       Similarly, the ion exchange resin can be regenerated for use with subsequent batches of polyethylene glycol containing chymosin by washing the ion exchange resin with a solution of water adjusted to the appropriate pH. For example, when a cation exchange resin is employed, it can be regenerated by washing it with a solution of water containing enough sulfuric acid to make the pH about 2.

25       The above aspects of this invention which enable the recycling of polyethylene glycol and the reuse of the ion exchange resins particularly lend the processes of this invention to efficient commercial and

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industrial operation for the purification of industrial quantities of chymosin, particularly microbially produced chymosin.

5        Having described the invention in general terms the invention can be better understood by reference to the following embodiments of the invention which are illustrated in the following examples. However the scope of this invention is to be  
10       determined by the appended claims, whereas the following examples are merely illustrative embodiments of particular ways in which the invention disclosed herein can be practiced.

#### EXAMPLES

15                                Example 1

         This example describes a chymosin recovery process using aqueous two phase polyethylene glycol extraction followed by contact with ion exchange resin to produce  
20       food grade chymosin. The chymosin is recovered from fermentation of an Aspergillus Niger var. awamori. The process is described in terms of 3000 l fermenter and a broth harvest volume of about 2500 l. When the  
25       fermentation is complete, the broth is inactivated by pH adjustment to 2.0-2.5 with sulfuric acid and addition of acetic acid. (See U.S. Patent Application Serial No. 07/365,945, filed June 13, 1989 by Lawlis et  
30       al., incorporated herein by reference.) The

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inactivation conditions are held for 1 hour at the fermentation temperature and air flow. This inactivation achieves sufficient viable cell reduction for containment to be broken.

5 After inactivation, the pH is maintained at 2.0-2.5. The inactivation will require about 125 kg of sulfuric acid and 25 kg of acetic acid.

The broth is extracted using a PEG 8000/sodium sulfate system at pH 2.0-2.5 by

10 diluting 3x with water followed by the addition of 4% wt/vol PEG 8000 (75kg) and 10.5% wt/vol anhydrous sodium sulfate. Upon mixing PEG/salt system with the broth, the

15 chymosin is extracted into the PEG phase in a short period of time. The two phase mixture is separated using an extraction centrifuge. The chymosin-rich, light PEG phase is collected for further processing. Using this

20 process the 2500 l of broth is diluted to about 7500 l for the PEG extraction and yields about 700 l of PEG phase extract.

The extract is diluted 3x with deionized water and filtered through cellulose pads

25 before the ion exchange step. The filtered extract is passed through a 10 l column of IBF Spherox SP cation exchange resin having a particle size of 40-100 microns. The loaded column is washed with 30 l of 6% NaCl solution

30 of pH 2. The column is eluted with 2M NaCl and 50mM phosphate buffer at pH 6.0. The



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capacity of the ion exchange resin is up to about 60g chymosin per 1 of resin. The eluted fraction containing the chymosin is collected for further processing. The column is  
5 regenerated for reuse by washing with water adjusted to pH 2 with sulfuric acid. The eluted chymosin solution is made 17% NaCl for commercial food grade use, or can be processed for other purposes, if desired.

10

Example 2

This example illustrated the high partition of chymosin into the PEG phase obtained by the process of this invention. Using the same fermentation broth of Example 1  
15 above, inactivated in the same way, the following extractions were performed on whole broth (Sample 1) and on a filtrate thereof from a rotary vacuum drum filter (Samples 2 and 3). All three samples were maintained at  
20 pH 2 for processing and were extracted with 5% PEG 8000 and 10% sodium sulfate wt/vol. Samples 1 and 2 were phase separated in a bottle centrifuge (RC3B) and Sample 3 was separated in an SA-1 continuous laboratory  
25 centrifuge.

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VOL. (1)	ACT. (CHU/1)	TOTAL CHYMOSIN (CHU)	RECOVERY (%)
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**SAMPLE 1:**

## 5 EXTRACTION OF WHOLE BROTH

RAW FEED	10	30	300	
TREATED FEED	15	13	195	
PEG Phase	1.7	206	350	117
REMAINDER	13	1.5	19	5

10                                MASS BAL =          122  
   K =          141

**SAMPLE 2:**

## EXTRACTION OF FILTRATE

15	RAW FEED	14	13.7	192	
	PEG PHASE	3.3	49	164	85
	REMAINDER	11.3	0.78	9	5

MASS BAL = 90  
K = 62

**SAMPLE 3:**

20                   EXTRACTION OF FILTRATE

RAW FEED	430	7.16	3078	
PEG PHASE	62	52.3	3127	104
REMAINDER	385	0.24	96	3

**25**

<b>MASS BAL =</b>	<b>107</b>
<b>K =</b>	<b>201</b>

(CHU - Chris. Hansen Unit -- 1 CHU/ml, under the following conditions:

30 Substrate: 110 g of low heat, spraydried  
skim milk powder is suspended  
in 1000 ml 0.05% calcium  
chloride. The milk is  
stirred for 30 minutes at  
room temperature and then  
35 left for rest for another 30  
minutes. The milk should be

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stored at a temperature between 4 and 25°C and not longer than 3 hours. The pH of the milk is about 6.5.

- 5           Temperature: 32°C plus or minus 0.2°C in a thermostatic water bath.
- 10           Enzyme addition: To 25 ml of the reconstitute skim milk is added 0.5 ml of enzyme solution, diluted to give a clotting time between 380 and 500 seconds,

will give a clotting time of 410 to 460 seconds)

- 15           The above mass balances are not 100% because the methods used for measurement of certain values are imprecise and provide approximate values. Nevertheless, the above tests illustrate the relative partitioning of the
- 20           chymosin achieved by this invention allowing for the experimental error of the test methods.

### Example 3

- 25           In this example of PEG phase similar to Example 1 diluted as indicated with deionized water and filtered was passed through a column of 1.0 ml of each resin, equilibrated in pH = 2.0 deionized water. Flow rate was 1.0 ml/min, washed with pH = 2.0 DI water. Eluted
- 30           with pH = 5.8, 2 M NaCl, 50 mM sodium phosphate (pi).

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## RESIN:

	IBF SP- SPHERODEX		PHARMACIA SP-SEPHA.		INDION SP-2		IBF SP- TRISACRYL	
5	DILUTION		DILUTION		DILUTION		DILUTION	
	1:2	1:4	1:2	1:4	1:2	1:4	1:2	1:4
	-----							
	LOADED (mgs)							
	34.3	34.3	34.3	34.3	34.3	34.3	34.3	17.6
10	UNBOUND (mgs)							
	9.8	3.82	26.5	0.312	34.8	17.6	27.3	0.338
	ELUTED (mgs)							
	26.5	26	8.84	21.2	0.32	1.05	10.8	17.3
	MASS BAL. (%)							
15	106	87	103	63	102	54	111	100

Example 4

20 This example is the same as Example 3 except the following was used for multiple elution: 670 mls SP-Spherodex Resin; load and elute @ 450 mls.min; load 1:4 dilution of PEG Extract pH = 2.0; wash with DI H<sub>2</sub>O pH 2.0, 2M NaCl pH 2.0; elute with 2 M NaCl, 200 mM Pi, pH 5.8.

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	#	Desc.	Vol.(1)	CHU/1	CHU
	1	START	240	11.07	2656.8
	2	VOID 1	170	0.69	117.3
5	3	VOID 2	60	7.21	432.6
	4	DI WASH	2.5	0	0
	5	NaCl WASH	7.4	4.89	36.186
	6	ELUT #1	1	1347	1347
	7	ELUT #2	1	924	924
10	8	ELUT #3	1	214	214
	9	ELUT #4	1	108	108
	10	ELUT #5	1	25.6	25.6
	11	ELUT #6	1	13.8	13.8
15	MASS BALANCE: 121.14				
	YIELD (% OF BOUND): 127.13				
	CAPACITY (MG/ML): 51.08				

Example 5

20 This example is the same as Example 4 except 860 mls PEG extract were treated with 1% "Nuchar SA" charcoal for 30 minutes, filtered, then diluted 1:3 and fed to a column of 2.5 mls resin having a bed depth of 3.2 cm. The results were as follows:

	#	DESC.	VOL.(1)	Mg/l	Mgs
25	1	Start	2,600	75	195
	2	Void	2,600	22.5	58.5
	3	DI	20	15.7	0.314
	4	Salt	30	137	4.11
30	5	Composite	36	3,667	132.0
	(280 CHU/ml)				

Mass Balance:  $\frac{194.9 \text{ g}}{195 \text{ g}} (100) = 99.9\%$

35 Yield of Bound:  $\frac{132 \text{ g}}{136.5 \text{ g}} (100) = 96.7\%$

Capacity:  $\frac{132 \text{ mgs}}{2.5 \text{ ml}} = 52.8 \text{ g/l}$

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EXAMPLE 6

In manner similar to the extraction procedure set forth in Example 2 above but using a pH of about 5.8, the enzymes listed below were extracted from an aqueous solution into the polyethylene glycol phase of the liquid-liquid 2 phase system to provide the following results (pure enzyme used):

10	<u>Enzyme</u>	<u>Protein (mg/ml)</u>	<u>Activity CHU/ml</u>	<u>Partition Coefficient</u>
	calf chymosin	1.7	28	87
	bovine pepsin	1.9	31	31.5
	porcine pepsin	2.3	29.7	1.45
15	E. parasitica aspartic protease	3.6	27	0.33
	M. miehei aspartic protease	2.4	20.5	0.24

This experiment was repeated but at this time at pH 2-2.5 with the following results:

20	<u>Enzyme</u>	<u>Protein (mg/ml)</u>	<u>Activity CHU/ml</u>	<u>Partition Coefficient</u>
	calf chymosin	1.2	89	>943
	bovine pepsin	1.3	50	>462
25	porcine pepsin	1.6	23.7	>206
	E. parasitica aspartic protease	2.5	15.2	1.18
30	M. miehei aspartic protease	1.7	48	0.94

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The above results indicate that while chymosin provides excellent partition coefficients, i.e., above about 85 at either pH 5.8 or 2-2.5, bovine pepsin and porcine pepsin provide excellent partition coefficients only at the lower pH. However, neither bovine pepsin nor porcine pepsin are polypeptides found in fermentation beer. On the other hand, the above data further indicates that microbial rennet, i.e., *E. parasitica* aspartic protease and *M. miehei* aspartic protease, do not possess significant partition coefficients at either the lower or the higher pH tested.

#### Example 7

The following example is a direct comparison of the partition coefficients at pH 2.5 and 5.5 for chymosin as well as for alpha-amylase, acid phosphatase, leu amino peptidase, and glucoamylase (GAM), found in the fermentation beer of Aspergillus Niger var. awamori. The extraction was conducted in the manner of Example 2 above with the exceptions noted below:

#### Chymosin

<u>Sample</u>	<u>Starting Conc. CHU/ml</u>	<u>Partition Coefficient</u>
pH 2.5 no cells <sup>a</sup>	7	98
pH 5.5 no cells	5.9	92
pH 2.5 with cells <sup>b</sup>	6.3	96
pH 5.5 with cells <sup>c</sup>	5.9	88

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Alpha Amylase

	Sample	Starting Conc. IU/ml	Partition Coefficient
5	pH 2.5 no cells <sup>a</sup>	19,400	approaches 0
	pH 5.5 no cells <sup>a</sup>	19,400	approaches 0
	pH 2.5 with cells <sup>b</sup>	19,400	approaches 0
	pH 5.5 with cells <sup>c</sup>	19,400	approaches 0

GAM

	Sample	Starting <sup>d</sup> Conc.	Partition Coefficient
10	pH 2.5 no cells <sup>a</sup>	3,777.5	approaches 0
	pH 5.5 no cells <sup>a</sup>	3,777.5	0.001451
	pH 2.5 with cells <sup>b</sup>	3,777.5	0.001988
	pH 5.5 with cells <sup>c</sup>	3,777.5	0.000980

15

Acid Phosphatase

	Sample	Starting Conc. IU/ml	Partition Coefficient
20	pH 2.5 no cells <sup>a</sup>	594	approaches 0
	pH 5.5 no cells <sup>a</sup>	594	approaches 0
	pH 2.5 with cells <sup>b</sup>	594	approaches 0
	pH 5.5 with cells <sup>c</sup>	594	approaches 0

leu amino peptidase

	Sample	Starting Conc. U/ml	Partition Coefficient
25	pH 2.5 no cells <sup>a</sup>	1,639	approaches 0
	pH 5.5 no cells <sup>a</sup>	1,639	0.000182
	pH 2.5 with cells <sup>b</sup>	1,639	approaches 0
	pH 5.5 with cells <sup>c</sup>	1,639	approaches 0

- 30 a = fermentation beer centrifuged to remove cells without first killing the cells  
 b = fermentation beer not centrifuged and cells are still alive  
 c = fermentation beer not centrifuged and cells are still alive
- 35 d = starting concentration reported in microgram glucose/ml/min. Starting conc. (all enzymes) were determined at pH 5.5.



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The above data demonstrates that while chymosin is partitioned into the polyethylene glycol phase, the other enzymes in the fermentation beer are not. Thus, this data establishes that chymosin is selectively partitioned into the polyethylene glycol phase when in the presence of fermentation polypeptides.

#### Example 8

A one liter aliquot of Aspergillus Niger var. awamori at pH 5.8 was centrifuged to remove cells (not killed) on a RC-3B centrifuge (Sorvall) at appropriately 5000 x g for about 15 minutes. The resulting fermentation broth was warmed to about 37°C. To this solution was sodium sulfate (10.5 wt to vol. percent) and PEG 8000 (4 wt. to vol. percent). The solution was mixed until the components dissolved. The solution was then subjected to centrifugation at 5000 x g (on the centrifuge) for about 15 minutes to enhance the separation of the resulting phases. The chymosin-rich polyethylene glycol phase (top phase) was separated from the salt-rich phase (bottom phase) by removing the bottom phase with a peristaltic pump. One-half of the top phase (45 ml) was diluted 1 to 3 with distilled water and loaded onto a 2.5 ml Pharmacia Q-sepharose column equilibrated with 50 mM sodium phosphate, pH 5.8. The column was washed with 14 column volumes of the same buffer. The chymosin was eluted with 50 mM

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sodium phosphate, pH 5.8, 2 M NaCl. The volume of eluant was 45 ml. The results are as follows:

	Vol. (in liters)	Act. (CHU/ml)	Total Chymosin	Percent Recov.	
5	Raw Broth	1	11.6	11,600	100
	Extract	0.0925	102.9	9,775 <sup>e</sup>	84
	Raffinate	0.925	0.53	490	4
	Q-seph.				
10	void	0.101	---	154	3
	Q-seph.				
	elute	0.045	75	3,375	69 <sup>f</sup>
	Overall recovery				58
15	Mass balance				69
	Partition Coefficient (K)			about 190 <sup>g</sup>	

20 e = as noted above, only one-half of the extract was used which means that only one-half of the chymosin (9775/2 or 4887 CHU) was applied to the anion exchange resin.

f = 69 % recovery is the yield achieved in comparison to the amount of chymosin found in the extract.

25 g = Partition coefficients of about 200 or more are difficult to measure accurately because of assay limitations. That is to say that because the partition coefficient is a ratio of the concentration of chymosin in the top phase divided by the concentration of the chymosin in the bottom phase and further because the amount of chymosin in the bottom phase is generally very small, small changes in this bottom concentration will produce large swings in the partition coefficient. Moreover, the concentration determined by assay methodology is particularly subject to variations at very low concentrations.

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35

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By following the procedures exemplified above, substantially pure chymosin is recovered. That is to say that the chymosin is at least about 90% by weight pure and preferably, at least about 95% by weight pure and can be prepared for commercial use without further significant treatment to remove impurities. The commercial chymosin product is usually diluted to about 5 grams per gallon or about 1.5 grams per liter chymosin. The salt (usually NaCl) concentration is normally brought up to about 18% and a preservative such as sodium benzoate is added. The final concentrated product intended for food grade use usually is also subjected to a final filtration to remove any undesirable solids or particles that may be present.

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## WHAT IS CLAIMED IS:

1           1. A method for recovering microbially  
2           produced chymosin from an aqueous fermentation  
3           beer additionally containing fermentation  
4           enzymes which comprises adding to the  
5           fermentation beer an effective amount of  
6           polyethylene glycol and an inorganic salt so  
7           as to form a two phase system, allowing the  
8           fermentation beer-polyethylene glycol-  
9           inorganic salt mixture to separate into a  
10          chymosin-rich, fermentation polypeptide-poor  
11          polymer phase and a chymosin-poor,  
12          fermentation polypeptide-rich salt phase, and  
13          recovering the chymosin-rich, fermentation  
14          polypeptide-poor polymer phase.

1           2. A method according to Claim 1 wherein  
2           the pH of the fermentation beer is about 6.5  
3           or less.

1           3. A method according to Claim 2 wherein  
2           the pH of the fermentation beer is about 3 or  
3           less.

1           4. A method according to Claim 3 wherein  
2           the pH of the fermentation beer is less than  
3           about 2.8.

1           5. A method according to Claim 1 wherein  
2           the average molecular weight of the  
3           polyethylene glycol is from about 600 to about  
4           12,000.

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1           6.    A method according to Claim 5 wherein  
2    the average molecular weight of the  
3    polyethylene glycol is from about 5,000 to  
4    about 10,000.

1           7.    A method according to Claim 1 wherein  
2    said inorganic salt is selected from the group  
3    consisting of sulfate salts and phosphate  
4    salts.

1           8.    A method according to Claim 7 wherein  
2    said inorganic salt is a sulfate salt.

1           9.    A method according to Claim 8 wherein  
2    said sulfate salt is selected from the group  
3    consisting of sodium sulfate, magnesium  
4    sulfate, and ammonium sulfate.

1           10.   A method according to Claim 1 wherein  
2    said aqueous fermentation beer is first  
3    filtered prior to addition of said  
4    polyethylene glycol and said inorganic salt.

1           11.   A method for recovering microbially  
2    produced chymosin from an aqueous fermentation  
3    beer additionally containing fermentation  
4    polypeptides which comprises adjusting the pH  
5    of the aqueous mixture to less than about 3,  
6    then adding to the fermentation mixture an  
7    effective amount of polyethylene glycol and an  
8    inorganic salt so as to form a two phase  
9    system, allowing the fermentation beer-

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10 polyethylene glycol-inorganic salt mixture to  
11 separate into a chymosin-rich, fermentation  
12 polypeptide-poor polymer phase and a chymosin-  
13 poor, fermentation polypeptide-rich salt  
14 phase, and recovering the chymosin-rich,  
15 fermentation polypeptide-poor polymer phase.

1 12. A method according to Claim 11 wherein  
2 the average molecular weight of the  
3 polyethylene glycol is from about 600 to about  
4 12,000.

1 13. A method according to Claim 12 wherein  
2 the average molecular weight of the  
3 polyethylene glycol is from about 5,000 to  
4 about 10,000.

1 14. A method according to Claim 11  
2 wherein said inorganic salt is selected from  
3 the group consisting of sulfate salts and  
4 phosphate salts.

1 15. A method according to Claim 14 wherein  
2 said inorganic salt is a sulfate salt.

1 16. A method according to Claim 15 wherein  
2 said sulfate salt is selected from the group  
3 consisting of sodium sulfate, magnesium  
4 sulfate, and ammonium sulfate.

1 17. A method according to Claim 11 wherein  
2 said aqueous fermentation beer is first

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3 filtered prior to addition of said  
4 polyethylene glycol and said inorganic salt.

1 18. A method for recovering and purifying  
2 microbially produced chymosin from an aqueous  
3 fermentation beer additionally containing  
4 fermentation polypeptides which comprises

5 a) adding to the fermentation beer an  
6 effective amount of polyethylene glycol and an  
7 inorganic salt so as to form a two phase  
8 system,

9 b) allowing the fermentation beer-  
10 polyethylene glycol-inorganic salt mixture to  
11 separate into a chymosin-rich, fermentation  
12 polypeptide-poor polyethylene glycol phase and  
13 a chymosin-poor, fermentation polypeptide-rich  
14 salt phase,

15 c) recovering the chymosin-rich,  
16 fermentation polypeptide-poor polyethylene  
17 glycol phase,

18 d) contacting the chymosin-rich,  
19 fermentation polypeptide-poor polyethylene  
20 glycol phase with an ion exchange resin under  
21 conditions wherein the chymosin binds to the  
22 resin and the polyethylene glycol passes  
23 through the resin; and

24 e) recovering the chymosin from the resin.

1 19. A method according to Claim 18 wherein  
2 the average molecular weight of the  
3 polyethylene glycol is from about 600 to about  
4 12,000.

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1           20. A method according to Claim 19 wherein  
2           the average molecular weight of the  
3           polyethylene glycol is from about 5,000 to  
4           about 10,000.

1           21. A method according to Claim 18  
2           wherein said inorganic salt is selected from  
3           the group consisting of sulfate salts and  
4           phosphate salts.

1           22. A method according to Claim 21 wherein  
2           said inorganic salt is a sulfate salt.

1           23. A method according to Claim 22 wherein  
2           said sulfate salt is selected from the group  
3           consisting of sodium sulfate, magnesium  
4           sulfate, and ammonium sulfate.

1           24. A method according to Claim 18 wherein  
2           said aqueous fermentation beer is first  
3           filtered prior to addition of said  
4           polyethylene glycol and said inorganic salt.

1           25. A method according to Claim 18  
2           wherein the pH of the fermentation beer is  
3           about 6.5 or less.

1           26. A method according to Claim 25  
2           wherein the pH of the fermentation beer is  
3           about 3 or less.



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1           27. A method according to Claim 26 wherein  
2           the pH of the fermentation beer is less than  
3           about 2.8.

1           28. A method according to Claim 18 wherein  
2           the pH of said chymosin-rich, fermentation  
3           polypeptide-poor polyethylene glycol phase is  
4           either from 5.0 to 6.5 or is about 3.0 or  
5           less.

1           29. A method according to Claim 28 wherein  
2           the pH of said chymosin-rich, fermentation  
3           polypeptide-poor polyethylene glycol phase is  
4           about 3.0 or less and a cation exchange resin  
5           is employed.

1           30. A method according to Claim 28 wherein  
2           the pH of said chymosin-rich, fermentation  
3           polypeptide-poor polyethylene glycol phase is  
4           from about 5.0 to about 6.5 and an anion  
5           exchange resin is employed.

1           31. A method according to Claim 18 wherein  
2           step a) is conducted at a pH of about 3 or  
3           less and after isolation of the polyethylene  
4           glycol phase, the pH of this phase is adjusted  
5           to from about 5.0 to 6.5 and an anion exchange  
6           resin is employed.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/03376

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>3</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 9/64

U.S.C1.: 435/226,815,816

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>4</sup>

Classification System <sup>1</sup>

Classification Symbols

U.S. 435/226,815,816  
426/36,42

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched <sup>5</sup>

COMPUTER SEARCH-APS, CA, DIALOG

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup>

Category <sup>*</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y	US, A, 4,144,130 (Kula, et. al.) 13 March 1979. See entire document.	1,2,5-10, 18-25
Y	US, A, 4,728,613 (Brewer et. al.) 01 March 1988. See especially column 2, line 50.	1,2,5-10,18-25
A	US, A, 4,743,551 (Subramanian) 10 May 1988.	1-31
A	US, A, 4,745,063 (Birschbach) 17 May 1988.	1-31

<sup>\*</sup> Special categories of cited documents: <sup>15</sup>

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search <sup>2</sup>

05 September 1990

Date of Mailing of this International Search Report <sup>2</sup>

07 NOV 1990

International Searching Authority <sup>1</sup>

ISA/US

Signature of Authorized Officer <sup>19</sup>

Charles Patterson